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Identification and Characterization of Linoleic Acid as an Endogenous Modulator of in Vitro *N*-1-Naphthylphthalamic Acid Binding

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An endogenous inhibitor of the in vitro binding of the phytotropin *N*-1-naphthylphthalamic acid to microsomal membranes was detected in extracts prepared from etiolated pea (*Pisum sativum* L.) epicotyls. Following extensive purification, the inhibitor was identified as linoleic acid. Authentic linoleic acid inhibited *N*-1-naphthylphthalamic acid binding noncompetitively in a dose-dependent manner, exhibiting a 50% inhibitory concentration of approximately 24 μM. Using a variety of fatty acids and their derivatives, this inhibition was found to exhibit strict structural requirements, with both linoleic and linolenic acids being the most inhibitory. A variety of membrane-solubilizing detergents elicited no such inhibitory activity when tested at equivalent concentrations. The possible physiological significance of this interaction is discussed and it is proposed that linoleic acid serves as an intracellular modulator of phytotropin binding and therefore polar auxin transport.

Historically, the integration and regulation of plant development has been ascribed to a limited number of endogenous PGRs (Guern, 1987). However, recent evidence has demonstrated that, as in other multicellular organisms, the coordination of plant development is achieved by an ever-increasing family of endogenous compounds with diverse biosynthetic origins (Davies, 1995).

In principle, new endogenous PGRs can be discovered by one of three approaches: (a) the discovery of novel pharmacological actions of synthetic exogenous compounds followed by the identification of their endogenous counterparts, (b) in vivo bioassay methods, and (c) in vitro receptor-based screens or assays. Most endogenous PGRs identified to date have been discovered using methods a and b. This is in stark contrast to the situation in metazoan physiology, in which receptor-based assays are routinely used to identify novel endogenous regulators and new agents (Sweetnam et al., 1993). Two examples of this type of approach include the identification of enkephalins as endogenous ligands for opiate receptors (Hughes et al., 1975) and the recent identification of the arachidonic acid derivative anandamide as an endogenous ligand for the cannabinoid receptor (Devane et al., 1992).

A prerequisite for the meaningful use of receptor-based screens is that the receptor be of physiological importance. This has been a major obstacle in plant studies in which information concerning the physiological significance of various PGR-binding proteins is either limiting or absent (Libbenga and Mennes, 1995). One exception to this situation is the phytotropin receptor (Lomax et al., 1995). Originally identified as the NPA-binding protein, the phytotropin receptor is well characterized both pharmacologically and physiologically. Although exact molecular details are lacking, it is clear that the phytotropin receptor is intimately involved in the polar transport of the auxin IAA (Lomax et al., 1995). Using a variety of synthetic compounds, a reasonably good correlation between in vitro binding activity and in vivo inhibition of polar auxin transport has been established (Katekar and Geissler, 1980).

Although originally identified as a receptor for various synthetic ligands, it has long been suspected that one or more endogenous ligands may interact with the NPA receptor and thereby modulate polar auxin transport (Thompson, 1971). Indeed, early studies demonstrated that crude plant extracts contained an unidentified factor(s) that partially inhibited NPA binding in in vitro assays (Hertel, 1981). The chemical nature and physiological relevance of this endogenous phytotropin-displacing material remains unknown. More recently, certain flavonoid aglycones have been shown to exhibit NPA-displacing activity in vitro and have been proposed as endogenous ligands for the phytotropin receptor (Jacobs and Rubery, 1988). However, it is entirely possible that other endogenous ligands exist.

Previous studies examining the effects of ethylene on polar auxin transport and phytotropin binding (Suttle, 1988a) showed that extracts prepared from etiolated pea (*Pisum sativum* L.) epicotyls contained one or more compounds that inhibited in vitro phytotropin binding (Suttle, 1988b). Preliminary studies demonstrated that this inhibitory material was not phenolic in nature and therefore was not a flavonoid. However, the exact chemical nature of this inhibitor was not known.

In this report the isolation and identification of linoleic acid as a major component of the inhibitory activity present

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Abbreviations: B_{max}, binding site density; Chaps, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; IC_{50}, inhibitor concentration for 50% displacement; K_d, binding affinity; NPA, *N*-1-naphthylphthalamic acid; PGR, plant growth regulator.

in pea extracts is reported. In addition, data exploring the biochemical basis for this inhibition as well as the structural requirements for activity are presented.

MATERIALS AND METHODS

Plant Material and Experimental Procedure

Untreated pea (*Pisum sativum* L. cv Alaska; Sunseeds, Twin Falls, ID; lot no. 40–019–020) seeds were surface-sterilized using a 1:4 (v/v) dilution of commercial bleach (5.3% sodium hypochlorite by weight). After soaking for 4 to 6 h in running deionized water, the seeds were sown in flats containing vermiculite and watered with tap water. Seedlings were used after 5 d of growth in the dark (27 ± 1°C). All experiments described in this paper were conducted at least twice. Where possible, individual treatments within an experiment were replicated (as indicated in each figure or table). Data from typical experiments are presented.

Microsome Preparation and NPA-Binding Assays

Microsomes were isolated from the upper portions of etiolated pea epicotyls (minus the plumule) by differential centrifugation, as described previously (Suttle, 1988a). The sedimented microsomes were resuspended in assay buffer (10 mM sodium citrate, 5 mM $MgCl_2$, 0.25 mM Suc, pH 5.0) at a tissue concentration of 2 g fresh weight equivalents mL^{-1}. Membranes were either used immediately or were frozen in liquid nitrogen and stored at −70°C until needed. Immediately prior to use, microsomes were diluted to a final tissue concentration of 0.4 g mL^{-1} with additional assay buffer.

NPA-binding assays were conducted on ice in assay buffer, as previously described (Suttle, 1988a). Each assay contained 0.94 pmol (0.046 μCi) of [^{3}H]NPA, 0 to 150 pmol of unlabeled NPA (Scatchard analysis), 1 μM carbonyl cyanide-*p*-trifluoromethoxyphenyl hydrazone, and microsomal membranes equivalent to 0.2 g fresh weight in a total volume of 1.5 mL. Nonspecific binding was defined using 10 μM unlabeled NPA. After a 60- to 75-min incubation period (4°C), separation of free from bound ligand was accomplished by filtration (GF/B filter, Whatman). Previous studies have established the validity and utility of filtration-based binding assays of in vitro NPA binding (Muday et al., 1993). Data analysis was performed using the programs EBDA and LIGAND (McPherson, 1985). Test compounds were prepared as acetone stocks, which were evaporated under a stream of nitrogen prior to assay.

Purification and Characterization of the Endogenous Inhibitor

A total of 1.2 kg (fresh weight) of etiolated epicotyls was harvested from 5- to 6-d-old pea seedings, washed in running deionized water, frozen in liquid nitrogen, and lyophilized. The extraction and initial purification steps were conducted in batch form using approximately 250 g fresh weight equivalents of dried tissue. During purification, activity was monitored using the in vitro NPA-binding assay described above. Active fractions were then pooled and subjected to the next round of purification.

The freeze-dried plant material was soaked for approximately 24 h in 80% (v/v) aqueous acetone, mechanically homogenized, clarified by filtration, and reduced to the aqueous phase by rotary film evaporation at 40°C. The aqueous extract was brought to 2 M with concentrated HCl, capped, and incubated at 100°C for 3 h. After cooling to room temperature, the hydrolyzed extract was partitioned once against an equal volume of hexane and the hexane fraction was discarded. The aqueous phase was then partitioned three times against equal volumes of ethyl acetate. The combined ethyl acetate fractions were dried by passage through a bed of anhydrous sodium sulfate and were reduced to a brown, oily residue by rotary film evaporation at 40°C. Aliquots of this residue were dissolved in 0.5 mM potassium-phosphate buffer (pH 8.2) and partitioned three times against diethyl ether. The ether phases were dried by passage through a bed of anhydrous sodium sulfate and reduced in volume by rotary film evaporation at 40°. At this stage, all of the ether phases were combined and reduced in volume, resulting in 0.81 g of a brown-red oil.

This active oil was then subjected to two rounds of purification by silica column chromatography. In the first round, the active oil was fractionated on a 50-g silica column using a step gradient of 0, 25, 50, 75, and 100% (v/v) ethyl acetate in hexane (100 mL/step). The active fractions (eluting between 25 and 75% ethyl acetate) were pooled and subjected to a second round of column chromatography using 30 g of silica and a step gradient of 0, 25, 50, 75, and 100% (v/v) ethyl acetate in benzene (100 mL/step). The active fractions (eluting between 25 and 50% ethyl acetate) were pooled and fractionated by reverse-phase chromatography using a column containing 15 g of preparative C_{18} bonded silica (Waters), and eluted with a step gradient of 0 to 100% acetonitrile in water (20% per step; 50 mL). The column was then washed with 100 mL of ethyl acetate. The active fractions (eluting at 100% acetonitrile and the ethyl acetate wash) were combined and reduced in volume to approximately 500 μL of an amber oil.

The active fractions were then further purified by three successive rounds of TLC using 500-μm silica GF plates (Analtech, Newark, DE). In the first round, aliquots of the active oil were redissolved in a minimum volume of ethyl acetate and fractionated by TLC using a mobile phase of hexane ethyl acetate:methanol (6:3:1 [v/v]). The active fractions (R_F 0.4–0.7) were then rechromatographed using a mobile phase of dichloromethane:acetone:acetic acid (9:1:0.1 [v/v]). Zones of activity (R_F 0.5–0.8) were fractionated a third time by TLC using a solvent system composed of dichloromethane:acetone:ammonia (9:1:0.1 [v/v]). In this TLC system, the activity was confined to the first zone (R_F 0.1). As a final TLC step, the active fraction was rechromatographed on a C_{18} reverse-phase plate (Analtech) using acetonitrile as the developing solvent. Activity was found at R_F 0.4 to 0.8. These fractions were combined and reduced in volume, yielding approximately 200 μL of a clear, yellow oil.

Prior to HPLC, the active oil was rechromatographed on a 1.5-g, C_{18} reverse-phase column that was eluted stepwise (5% increments) with a gradient of tetrahydrofuran in water (10 mL/step). The active fractions (eluting between 45 and 65% tetrahydrofuran) were combined, taken to near-dryness, and redissolved in 0.5 mL of tetrahydrofuran. After filtering through a 0.22-μm filter, the extracts were fractionated by reverse-phase HPLC using a C_{18} column (PrepPak, Waters) eluted with a 7-min linear gradient of 75 to 100% (v/v) acetonitrile in 1% (v/v) acetic acid (5 mL/min). Activity eluted between 20 and 22 min.

These fractions were dried under a stream of nitrogen (40°C), derivatized with diazomethane, and subjected to MS with a gas chromatograph/mass spectrometer (model 5992A, Hewlett-Packard) with a 25-m fused silica capillary column coated with methyl silicone (0.52-μm thick), which was programmed from 150 to 300°C at 10°C min^{-1}. The ionization potential was 70 eV.

Chemicals

[2,3,4,5-^{3}H]NPA (49 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled NPA was obtained from Pfaltz and Bauer (Waterbury, CT) and was purified by recrystallization prior to use. Linoleic acid and all other fatty acids and their derivatives were purchased from Nu Chek Prep (Elysian, MN). All other chemicals used were of the highest grade commercially available.

RESULTS

Acidic, ethyl acetate-soluble fractions prepared from etiolated *P. sativum* epicotyl extracts contained one or more substance(s) capable of reducing NPA binding to microsomes in a dose-dependent manner (Fig. 1, dashed line). Similar extracts prepared from acid-hydrolyzed *P. sativum* extracts exhibited increased NPA-displacing activity per unit tissue equivalent (Fig. 1, solid line).

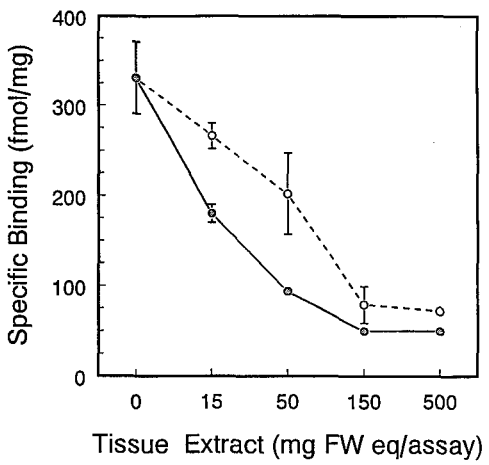

Figure 1. Inhibition of specific in vitro NPA binding to microsomes by extracts (○) and acid-hydrolyzed extracts (●) prepared from etiolated *P. sativum* epicotyls. When larger than the symbol, bars indicate SD. FW, Fresh weight.

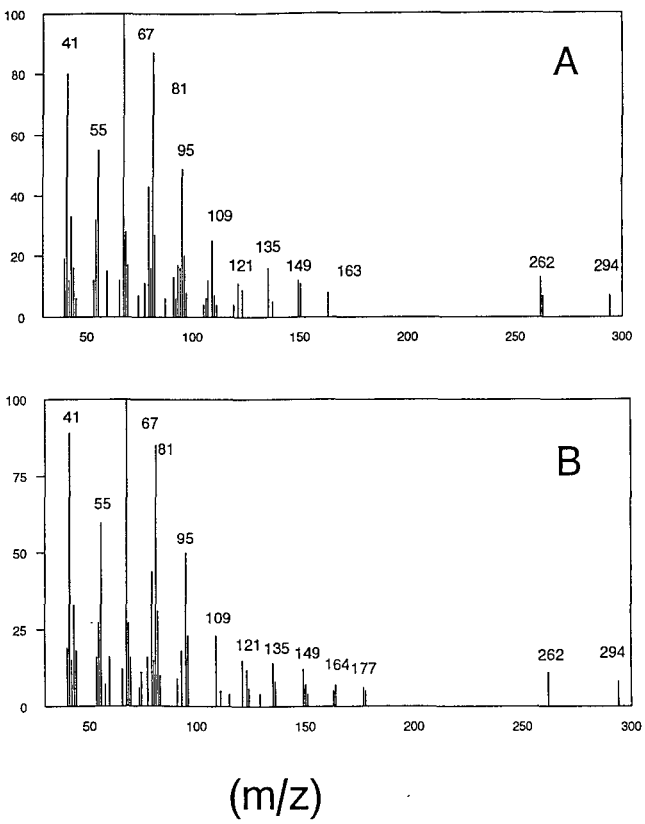

Figure 2. Mass spectra of the methyl esters of the endogenous inhibitor (A) and authentic linoleic acid (B). Both compounds exhibited a retention time of 8:24 min.

Previous studies demonstrated that the NPA-displacing substance(s) present in these extracts is a heat-stable, low-molecular-weight (approximately 300) compound of low polarity, which contains a free carboxylic acid group (Suttle, 1988b). Based on these and other criteria, a purification scheme was devised (see "Materials and Methods"). An acid-hydrolyzed extract prepared from a total of 1.2 kg (fresh weight) of etiolated *P. sativum* epicotyls was subjected to this purification scheme. Fractions containing NPA-displacing activity in in vitro binding assays were pooled and further purified. The NPA-displacing activity eluted as a single peak following reverse-phase HPLC (data not presented). The active fractions were then methylated and subjected to GC-MS analysis. A single major peak with a retention time of 8:24 min was observed, which yielded the mass spectrum presented in Figure 2A. A molecular ion (M^+) of 294 was also observed, as were other prominent ions at *m/z* 262, 149, 135, 109, 95, 81, 67 (base peak), 55, and 41. The sequential loss of 14 mass units from many of these peaks was reminiscent of a spectrum of a fatty acid derivative. Following a brief survey of the common fatty acids, it was discovered that the methyl ester of linoleic acid exhibited an identical retention time on GC and exhibited a near-identical mass spectrum (Fig. 2B).

To confirm the identity of the endogenous NPA-displacing substance as linoleic acid, the ability of authentic linoleic acid to inhibit in vitro NPA binding was tested.

When added to the standard binding assay, authentic linoleic acid inhibited saturable NPA binding in a dose-dependent manner (Fig. 3). An IC_{50} value of approximately 24 μM for linoleic acid was observed.

The inhibition of NPA binding by linoleic acid could have been the result of either a reduction in K_d, B_{max}, or both. The nature of the observed inhibition was examined by Scatchard analysis (Scatchard, 1949) of saturation equilibrium binding data. In untreated microsomes, saturable binding of NPA was consistent with a single class of binding sites and exhibited a K_d of 22 nM and a B_{max} of 0.44 pmol/assay (Fig. 4). In the presence of 24 μM linoleic acid, NPA binding was also consistent, with a single class of binding sites that exhibited a K_d of 25 nM and a B_{max} of 0.20 pmol/assay.

The specificity of this interaction was determined by examining the in vitro efficacy of various fatty acids and fatty acid derivatives structurally related to linoleic acid to inhibit saturable NPA binding. The fully saturated and monounsaturated 18-carbon fatty acids (stearic, oleic, and elaidic acids) were essentially inactive in this assay (Table I). Methylation or reduction of the carboxylic acid function of linoleic acid resulted in a substantial loss of activity. Reorientation of the two double bonds to the *trans,trans* configuration (linoelaidic acid) resulted in a loss of activity. This was also the case when the position of one of the double bonds was altered (conjugated linoleic acid). Shifting the position of the double bonds without altering the *cis,cis* configuration (nonadecadienoic acid) resulted in a partial loss of inhibitory activity. The triply unsaturated fatty acid (linolenic acid) was as potent as linoleic acid. As before, altering the position of one of the three double bonds (γ-linolenic) resulted in a loss of binding activity. Finally, extension of the chain length to 20 carbon atoms (arachidonic acid) also reduced the inhibitory activity.

Due to its amphipathic nature, it is possible that the inhibitory activity of linoleic acid on NPA binding could be due to a detergent-like action. To examine this possibility more closely, the effects of several well-known membrane-solubilizing detergents on in vitro NPA binding were directly compared with linoleic acid (Table II). As expected, inclusion of 30 or 100 μg of linoleic acid in the in vitro binding assay resulted in a 71 or 87% inhibition of saturable NPA binding, respectively. Inclusion of the membrane-active detergents octylglucoside or Chaps at 30 or 100 μg/assay had little or no effect on saturable NPA binding. Inclusion of Triton X-100 at 30 or 100 μg/assay resulted in a 30 and 49% stimulation of saturable NPA binding, respectively.

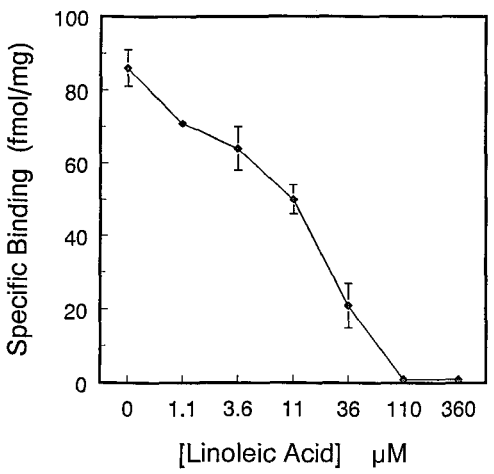

Figure 3. The effects of increasing concentrations of authentic linoleic acid on the specific in vitro binding of NPA to microsomes. Bars indicate SE (n = 3).

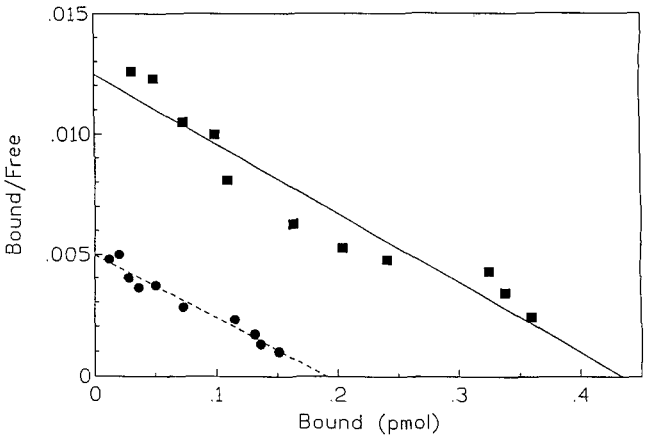

Figure 4. Scatchard analyses of equilibrium [^{3}H]NPA binding to microsomes prepared from etiolated *P. sativum* epicotyls in the absence (■) and presence (●) of 24 μM linoleic acid.

DISCUSSION

Using a receptor-based screen, linoleic acid was identified and characterized as an endogenous modulator of phytotropin binding in etiolated pea epicotyls. To be physiologically relevant, the binding of a ligand to a receptor should exhibit both high affinity and structural specificity. In competition-binding studies using NPA as the labeled ligand, linoleic acid exhibited an IC_{50} value of approximately 24 μM (Fig. 3). The structural requirements for NPA-displacing activity were quite strict, with only minor changes in chemical structure resulting in pronounced diminution of in vitro displacing activity (Table I). Of the fatty acid derivatives examined, only linolenic acid exhibited comparable in vitro activity. Thus, linoleic acid displayed both high affinity and structural specificity in this assay.

Although linoleic acid was isolated and identified in acid-hydrolyzed extracts, it is important to note that inhibitory activity was also present in nonhydrolyzed samples. Thus, the presence of the inhibitory activity cannot be strictly a hydrolytic artifact. Furthermore, as can be seen from the data presented in Figure 1, nonhydrolyzed extracts prepared from tissue samples equivalent to approximately 100 mg fresh weight contained sufficient amounts of inhibitory activity to displace 50% of the NPA specifically bound to microsomes prepared from approximately 200 mg fresh weight of tissue (see "Materials and Methods"). Thus, endogenous inhibitory activity was present in

Table I. *Effects of various fatty acids and their derivatives (all at 10 μg/mL) on specific binding of [^{3}H]NPA to microsomal membranes isolated from etiolated pea epicotyls*

Compound	Chain Length	Double Bond Position	Configuration	Specific Binding[a]	Control
				fmol/mg	%
Control	–[b]	–	–	176 ± 9	100
Stearic	18	–	–	148 ± 7	84
Oleic	18	9	*c*[c]	166 ± 6	94
Elaidic	18	9	*t*	157 ± 12	89
Linoleic	18	9, 12	*c,c*	59 ± 5	33
Methyl linoleate	18	9, 12	*c,c*	116 ± 3	66
Linoleic alcohol	18	9, 12	*c,c*	113 ± 7	64
Linoelaidic	18	9, 12	*t,t*	145 ± 6	82
Conjugated linoleic	18	9, 11	*t,t*	145 ± 3	82
Nonadecadienoic	19	10, 13	*c,c*	103 ± 4	59
Linolenic	18	9, 12, 15	*c,c,c*	55 ± 0	31
γ-Linolenic	18	6, 9, 12	*c,c,c*	90 ± 7	51
Arachidonic	20	5, 8, 11, 14	*c,c,c,c*	122 ± 6	69

[a] Mean ± SE (n = 3). [b] –, Not applicable. [c] *c, cis; t, trans.*

levels sufficient to exert a noticeable effect on NPA binding (and presumably action). This observation is also consistent with a physiological role for this inhibitor in this tissue.

Although the physiological relationship between phytotropin binding and polar auxin transport is well established, the molecular details of this interaction are presently unknown (Lomax et al., 1995). Based on competition studies, distinct binding sites for transportable auxins, phytotropins, and nonphytotropin transport inhibitors have been proposed (Rubery, 1987). In addition, it is not clear if all of these binding sites exist on one or more proteins (Morris et al., 1991). Scatchard analysis of [^{3}H]NPA binding in the presence and absence of linoleic acid (Fig. 4) demonstrates that the fatty acid inhibition of phytotropin binding is noncompetitive, and suggests that these two ligands bind to distinct sites. Thus, linoleic acid cannot be considered to be a phytotropin analog, but rather a modulator of phytotropin binding.

Noncompetitive inhibition of single substrate reactions (such as ligand binding) is relatively unusual. Mechanistically, this type of inhibition can be viewed as an all-or-none response. Thus, the binding of linoleic (or linolenic) acid results in the inactivation of the NPA binding site(s). At any given concentration of linoleic acid, a proportion of NPA-binding sites would be unavailable for binding, whereas the remaining sites would be unaffected, yielding the observed reduction in B_{max} without a corresponding effect on K_d. The protein-modifying agents *N*-ethylmaleimide (an alkylating agent) and iodine (an oxidizer) both elicit noncompetitive inhibition of NPA binding (Thein and Michalke, 1988). However, the relative unreactivity of linoleic acid, coupled with the strict structural specificity observed (Table I), argue against a similar mechanism for the inhibition caused by linoleic acid.

Is the in vitro activity the result of a detergent-like action? The available evidence suggests that it is not. First, the above-mentioned structural specificity would not be expected if membrane solubilization/perturbation were the underlying cause of this effect. Also, the inclusion of known membrane-solubilizing agents such as octylglucoside or Chaps at comparable concentrations had no such inhibitory effect (Table II); in fact, the addition of one widely used detergent (Triton X-100) actually stimulated NPA binding. This stimulatory effect by certain detergents has been observed previously by others (Sussman and Gardner, 1980).

Similarly, an oxidation product formed spontaneously or enzymatically from linoleic acid is unlikely to be responsible for the observed activity. First, to minimize auto-oxidation artifacts, fresh solutions of linoleic acid (and the other fatty acids) were prepared on the day of use from pure stocks maintained under nitrogen at −20°C. Enzymatic oxidation catalyzed by a contaminating lipoxygenase also appears unlikely, since the in vitro activity of various fatty acid derivatives does not correspond to their ability to serve as lipoxygenase substrates. The basic structural requirement for lipoxygenase substrates is a *cis,cis*-1,4-pentadiene moiety (Vick, 1993). Thus, several compounds, such as nonadecadienoic, γ-linolenic, and arachidonic acids, which exhibit only marginal NPA-displacing activity (Table I), can serve as lipoxygenase substrates. In addition, methylation of lino-

Table II. *Effect of linoleic acid and various detergents on specific binding of [^{3}H]NPA to microsomal membranes isolated from etiolated pea epicotyls*

Compound	Concentration	Specific Binding[a]	Control
	μg/assay	*fmol/mg*	%
None	–[b]	354 ± 33	100
Linoleic acid	30	104 ± 27	29
	100	46 ± 30	13
Octyl glucoside	30	288 ± 9	81
	100	349 ± 78	99
Chaps	30	354 ± 0	100
	100	337 ± 48	95
Triton X-100	30	462 ± 42	131
	100	528 ± 27	149

[a] Mean ± SD. [b] –, Not applicable.

leic acid results in a dramatic decrease in in vitro displacing activity, but typically has little effect on its susceptibility to lipoxygenase-catalyzed peroxidation.

The roles of free fatty acids and their derivatives as integral components of various signal transduction pathways are well established in metazoan physiology (Exton, 1994; Liscovitch and Cantley, 1994). In plants free fatty acids elicit a number of interesting effects both in vivo and in vitro. Continuous exposure of seeds to short-chain fatty acids inhibits germination, whereas a brief exposure to certain of these fatty acids can sensitize seeds to the germination-stimulating effects of exogenous ethylene (Berrie et al., 1975; Whitehead and Nelson, 1992). Both linoleic and linolenic acids stimulate the abscission of bean petiole explants (Ueda et al., 1991). Recently, it has been shown that treatments of soybean cells with linoleic acid results in pronounced changes in cytoskeletal organization (Grabski et al., 1994). In vitro, free fatty acids elicit a number of effects. Unsaturated fatty acids (including linoleic acid) activate a highly purified, calcium-dependent protein kinase in wheat, whereas their saturated analogs are much less effective (Lucantoni and Polya, 1987). Both linoleic and linolenic acids stimulate the activity of the plasma membrane H^+-ATPase (Palmgren et al., 1988).

Although the existence of an endogenous phytotropin-like substance was originally proposed during initial studies on NPA binding in plant tissue extracts (Thompson, 1971), the existence and identity of endogenous ligands and/or allosteric effectors (modulators) of this receptor remain an enigma. To date, only a limited number of endogenous compounds have been shown to inhibit in vitro NPA binding. These compounds include certain flavonoid aglycones and their sulfated derivatives (Jacobs and Rubery, 1988; Faulkner and Rubery, 1992), the stilbene hydrangeic acid and its saturated analog lunularic acid (Katekar et al., 1993), and the di- and trienoic free fatty acids (this study). In addition, both potato tubers and the nodulating bacterium *Rhizobium meliloti* produce one or more unidentified factors that inhibit in vitro NPA binding (Hertel, 1981; Hirsch et al., 1993). Of the compounds identified to date, only the flavonoid aglycones and the free fatty acids have sufficiently broad distribution in the plant kingdom to be considered as general effectors of NPA binding.

The IC_{50} value calculated for linoleic acid (approximately 24 μM; Fig. 3) is similar to that (approximately 10 μM) reported for quercetin, one of the more active flavonoid aglycones (Jacobs and Rubery, 1988). The inhibitory activity of both types of compounds exhibits relatively strict structural requirements (Table I) (Jacobs and Rubery, 1988). In contrast to the flavonoid aglycones, linoleic acid inhibits NPA binding in a noncompetitive manner (Fig. 4), suggesting that it works by acting at a site distinct from that occupied by NPA or the flavonoids. Thus, linoleic acid cannot be viewed as an endogenous NPA analog or phytotropin, but rather as a modulator of NPA-binding activity.

Like the flavonoid aglycones, free linoleic acid is thought to be present in cells at low concentrations under normal conditions. Liberation of the active free acid could occur by the action of various lipolytic enzymes present in all cells. One such enzyme, phospholipase A_2, specifically releases fatty acids from the *sn*-2 position of the glycerol backbone. This position corresponds to the typical position for unsaturated fatty acids such as linoleic acid. In animal and possibly plant cells as well, phospholipase A_2 participates in various signal transduction pathways and is therefore sensitive to a variety of biotic and abiotic signals (Scherer, 1996). Thus, liberation of free fatty acids by enzymatic hydrolysis could link the perception of various stimuli with alterations in the activity of the polar auxin transport system. For example, in certain tissues, ethylene treatment results in an acceleration of membrane lipid catabolism catalyzed by an unidentified acyl hydrolase (thereby releasing free fatty acids) (Suttle and Kende, 1980). In addition, ethylene treatment often results in a decline in polar auxin transport and a decrease in specific NPA binding to microsomes, which, in turn, is the result of a decrease in NPA B_{max}, with no change in apparent K_d (Suttle, 1988a).

In conclusion, linoleic acid has been identified as a potential endogenous modulator of phytotropin binding in etiolated pea epicotyls. This interaction exhibits both high (micromolar) affinity and strict structural requirements. It is proposed that linoleic acid serves as an intracellular modulator of the activity of the auxin transport system. This intracellular site of action corresponds to the predominant site of fatty acid abundance in most cells (membranes), the localization of an enzyme activity capable of releasing the free (active) fatty acid (phospholipase A_2), and the presumptive (plasmalemma) localization (Bernasconi et al., 1996) of the phytotropin receptor. In light of these considerations, further study of the physiological significance of this interaction seems warranted.

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